JACOBS PUBLISHERS

OPEN ACCESS

Jacobs Journal of Biomarkers

Research Article

Wnt and Notch Signaling Pathways are Involved in the Development of Aggressive Salivary Gland Neoplasms

UK Sinha MD[1], O. Gantz BS[1], J. Brooks MD[1], R Masood Ph D[1*]

[1]*Department of Otolaryngology – Head and Neck Surgery, University of Southern California, Keck School of Medicine, Los Angeles, CA, USA*

**Corresponding author: Dr. Rizwan Masood, University of Southern California, Hoffman Medical Research 903,2011 Zonal Avenue,Los Angeles, California 90089-9300,Tel: (323)-442-2449; Email: masood@med.usc.edu*

Received: 10-16-2015

Accepted: 10-28-2015

Published: 11-04-2015

Abstract

Purpose

Salivary gland tumors are uncommon, however, the aggressive subtypes portend poor prognoses due to growth patterns, wide spread invasion, high incidence of local recurrence and distant metastases. An advance in the understanding of tumorigenesis is imperative to determine potential targets for therapy.

Methods

Twenty five patients with diagnosed salivary gland tumors were retrospectively entered into the study. Immunohistochemistry and H&E staining were performed on the tumor specimens including oncocytic cystadenoma (OC), pleomorphic adenoma (PA), acinic cell carcinoma (ACC), adenoid cystic carcinoma (AdCC), and high grade mucoepidermoid carcinoma (MEC). Four genetic markers were tested based on microarray results-TGF-β, Frizzled-6 (FZD6), Delta-4, and β-catenin. The degree of expression was derived from microscopic images using ProImage software.

Results

TGF-β and β-catenin were readily expressed in each salivary gland neoplasms, but showed greater induction in aggressive neoplasms. β-catenin exhibited increased nuclear staining in aggressive neoplasms, particularly ACC. FZD6 and Delta-4 were highly expressed in MEC, ACC and AdCC respectively but minimally expressed in the benign tumor types.

Conclusion

TGF-β, FZD6 and Delta-4 appear to play a larger role in tumorigenesis of aggressive subtypes (ACC, AdCC, MEC) of sali-

vary gland tumors as compared to slower-growing tumor types (OC, PA). β-catenin exhibits increased nuclear staining in more aggressive tumors. FZD6 and β-catenin proteins are involved in the Wnt signaling pathway, while Delta-4 is involved in Notch signaling; thus these pathways are implicated in growth patterns of aggressive salivary gland neoplasms.

Keywords: Salivary Gland Neoplasms; Wnt Signaling Pathway; Notch Signaling Pathway

Introduction

Tumors of the salivary glands are relatively uncommon malignancies, representing only 11% of oropharyngeal neoplasms in the United States [1]. Salivary gland tumors consist of over 20 different histological subtypes [2] and can affect both the major salivary glands (parotid, submandibular, sublingual), as well as minor salivary glands throughout the oral cavity and oropharynx. Salivary tumors present with a wide range of biological behavior, from benign (oncocytoma, cystadenoma, pleomorphic adenoma) to highly aggressive (low grade acinic cell carcinoma, adenoid cystic carcinoma, and high grade mucoepidermoid carcinoma) with wide spread invasion, local recurrence, and distant metastases.

A number of investigators have attempted to identify specific gene expression patterns that correlate with tumor aggressiveness. There has been significant focus on genes related to cell-cell adhesion, as it is thought that the loss of adhesion mediators leads to tumorigenesis, invasion, and metastasis. Some studies have shown that E-cadherin expression is reduced in a subset of malignant tumors compared to benign salivary tumors [3]. E-cadherin has been implicated in contributing to tumorigenicity by promoting migration, invasion, adhesion, and angiogenesis [4]. Abnormal expression of β-catenin, a protein associated with E-cadherin, has also been shown to be of prognostic importance in some types of salivary tumors such as mucoepidermoid carcinoma [5].

The Wnt and Notch signaling pathways have been studied extensively because of their role in cancer biology. Dysregulation of these pathways has been implicated as important mechanisms in the development and progression of many cancers, including breast, colon, hepatocellular, and lung [6]. Components of these pathways, particularly β-catenin and E-cadherin in Wnt signaling, and Notch and its ligand Delta, are of particular interest for their role as potential targets for gene therapy.

Notch signaling plays a role in stem cell biology, tumor formation, angiogenesis and cell death. Notch-4 has been studied earlier in salivary adenoid cystic carcinoma and showed to play an important contribution to the metastasis of this disease [7]. Recently, the expression and significance of notch members signaling pathway were studied in adenoid cystic carcinoma and showed elevated expression levels of all three members of Notch family along with the ligands (Jagged-1 and Delta) [8].

Salivary gland tumors present with a wide range of clinical behaviors, and understanding the biochemical profiles of the different subtypes of tumors is an important step in developing effective, targeted treatments. Continued research is needed to further elucidate the role of the Wnt and Notch signaling pathways in the tumorigenesis and development of aggressive salivary tumors, and holds the potential for earlier detection of aggressive tumors as well as novel therapeutic approaches.

This current study was developed to investigate the role of the Wnt and Notch signaling pathways, including Frizzled-6, β-Catenin, TGF-β and Delta-4 in tumorigenesis and the development of aggressive salivary gland neoplasms compared to more benign histological subtypes.

Materials and Methods

Patient Selection

Twenty five adult patients from the Keck Hospital of the University of Southern California with diagnosed salivary tumors were retrospectively entered into the study. The study was approved by the appropriate institutional review board at the Keck Hospital of University of Southern California (USC).

Because of the rare nature of salivary tumors this project was limited by our relatively small sample size taken from a single tertiary care institution. Our tumor samples were collected over a 2 year period from 2011 to 2013.

Demographics

The patients entered into the study for benign and aggressive tumors were demographically similar. The median age of patients enrolled was 58 for aggressive tumors and 54 for benign tumors. The age range was also similar, 32 – 83 years old for aggressive tumors and 36 – 74 for benign tumors. The male to female ratio was 5:1 for aggressive tumors and 4:1 for benign tumors (Table 1).

	Aggressive (High and low grade)	Benign
Total Number	15	10
Median Age, y	58	54
Age Range, y	32 – 83	36-74
Gender Ratio (M:F)	5:1	4:1

Table 1. Clinicopathological characteristics. Aggressive tumors include adenoid cystic carcinoma, low grade acinic cell carcinoma, and high grade mucoepidermoid carcinoma. Benign tumors include pleomorphic adenoma and oncocytic cystadenoma.

Immunohistochemistry

Immunohistochemistry and H&E staining were performed

on 5 tumor specimens in patients with various salivary gland tumors, including adenoid cystic carcinoma (AdCC), low grade acinic cell carcinoma (ACC), high grade mucoepidermoid carcinoma (MEC), oncocytic cystadenoma (OC), and pleomorphic adenoma (PA). Four genetic markers were tested based on microarray results such as -TGF-β, Frizzled-6 (FZD6), Delta-4, and β-catenin.

Immunohistochemical detection for four antibodies was performed on formalin-fixed, paraffin-embedded sections (4 μm) (Table 2). The different concentration of antibodies were used in the immunohistochemistry and shown in the Table 2. After 10% formalin fixation, sections were blocking with 3% H2O2 and blocking buffer (0.1% gelatin, 0.1% BSA, 2.5% donkey serum and 2.5% goat serum in 0.3% triton-PBS) then incubated with primary antibodies at 4°C for overnight. Followed with polyhorseradish peroxidase (HRP) anti-rabbit or mouse IgG reagent to localize the primary antibody, and diaminobenzidine (DAB) was used to visualize the complex. Then the sections were counterstained with hematoxylin, dehydrated, cleared, and mounted. Negative controls included replacement of the primary antibody with rabbit, goat or mouse IgG of the same species. The degree of expression of each gene was derived from microscopic images, using ProImage software to determine %IOD and fold induction.

Primary Antibody	Manufacturer	Dose/Dilution factor	Incubation Time
Delta-4	Polyclonal, Santa Cruz Biotechnology, Dallas, TX, USA	4 μg/ml / 50	Overnight
TGF-β	Polyclonal, Santa Cruz Biotechnology, Dallas, TX, USA	2.5 μg/ml / 80	Overnight
Frizzled-6	Polyclonal, Novus Biologicals, Littleton, CO, USA	4 μg/ml / 25	Overnight
β-Catenin	Polyclonal, Sigma Aldrich, St. Louis, MO, USA	1 μg/ml / 1000	Overnight

Table 2. This table represents the manufacturers of specific antibodies, dilution factors, and incubation times.

Results

Immunohistochemical analysis was performed in all tumor samples. The intensity of staining of tumor samples was compared to negative control tissue (Figures 1-5). It was observed that TGF-β, β-catenin, FZD-6, and Delta-4 were readily expressed with different expression levels in each salivary gland neoplasm.

As shown on figure 1, in oncocytic cystadenoma, β-Catenin, Delta-4, and FZD-6 were all slightly upregulated from baseline levels. Figure 2 demonstrate similar results in pleomorphic Adenoma however there was a minor increase in TGF-β. Contrary to figure 1 and 2, figure 3, 4 and 5 demonstrate a drastic increase in β-Catenin, Delta-4, and FZD-6 in low grade Acinic Cell Carcinoma, Adenoid Cystic Carcinoma and Mucoepidermoid carcinoma. These three forms of salivary cancer have all been shown to be more malignant.

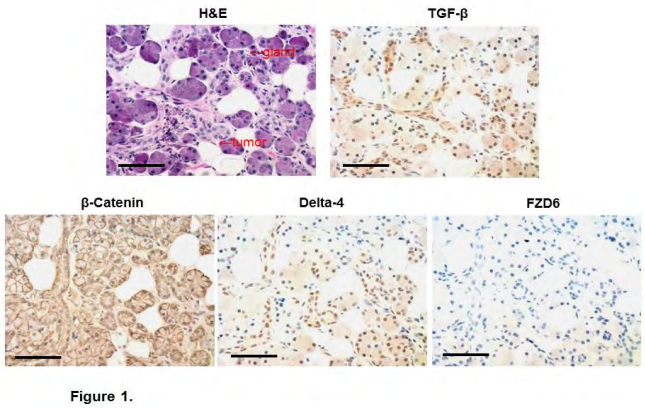

Figure 1. H&E stain, negative control, and immunohistochemistry in Oncocytic Cystadenoma (OC). Scale bar=100 μm.

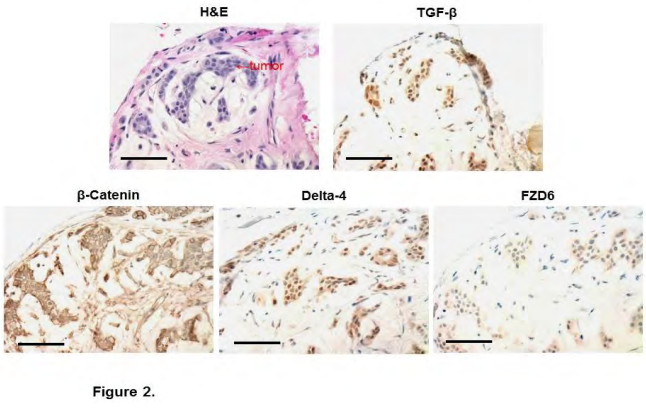

Figure 2. H&E stain, negative control, and immunohistochemistry in Pleomorphic Adenoma (PA). Scale bar=100 μm.

It was observed that TGF-β, FZD-6, and Delta-4 had significantly higher fold induction in aggressive neoplasms compared to benign neoplasms (Table 3). AdCC, ACC and MEC demonstrated greater induction of Delta-4 and FZD6 compared to other benign neoplasms, while acinic cell carcinoma showed the greatest expression of FZD-6. TGF-β had relatively higher induction among the aggressive neoplasms compared to benign.

	Benign Neoplasms		Aggressive Neoplasms		
	OC	PA	ACC	AdCC	MEC
TGF-β	5	8	15	12	25
β-Catenin	10	11	22	19	10
Delta-4	4	4	16	15	18
FZD-6	1	2	32	10	21

Table 3. Staining intensity of expression, based on fold induction, of each marker in different benign and aggressive tumors. OC = Oncocytic Cystadenoma, PA = Pleomorphic Adenoma, ACC = low grade Acinic Cell Carcinoma, AdCC = Adenoid Cystic Carcinoma, MEC = high grade Mucoepidermoid Carcinoma.

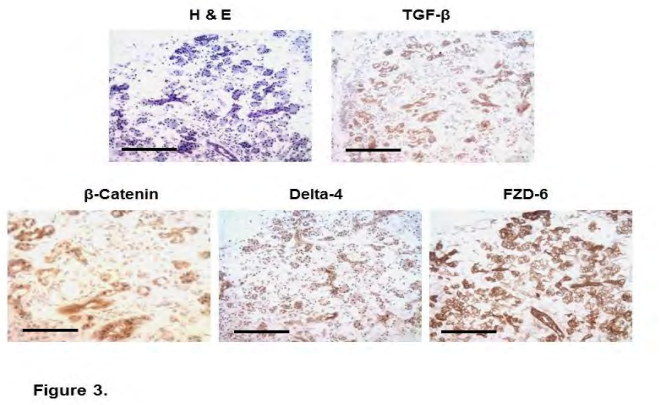

Figure 3. H&E stain, negative control, and immunohistochemistry in low grade Acinic Cell Carcinoma (ACC). Scale bar=100 μm.

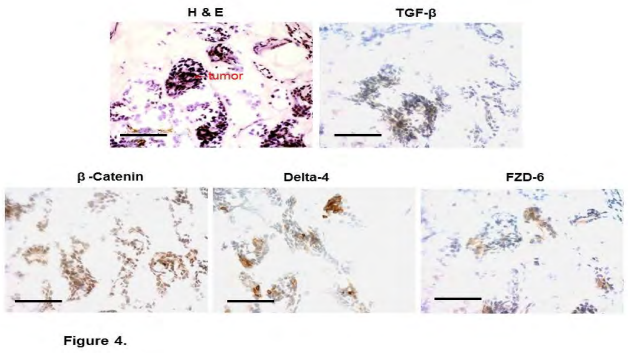

Figure 4. H&E stain, negative control, and immunohistochemistry in Adenoid Cystic Carcinoma (AdCC). Scale bar=100 μm.

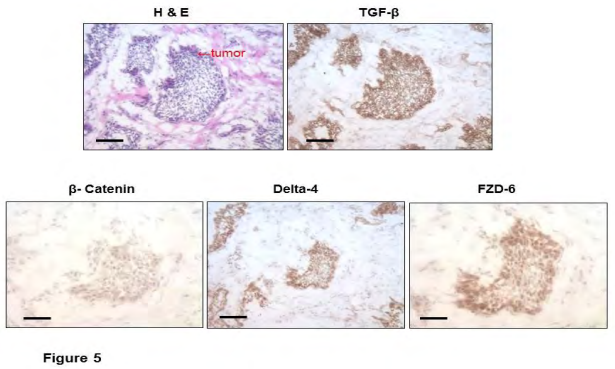

Figure 5. H&E stain, negative control, and immunohistochemistry in high grade Mucoepidermoid Carcinoma (MEC). Scale bar=100 μm.

β-catenin showed similar levels of induction among all tumor samples, however it in exhibited increased nuclear staining in aggressive neoplasms, particularly ACC. Staining was limited to the cytoplasm in the benign samples.

Discussion

The Wnt cell signaling pathway is an important regulator of cell adhesion and development, and is involved in integrating signals from other cellular pathways, including TGF-β. Several publications have studied the involvement of the Wnt pathway and TGF-β on the regeneration of salivary glands following radiotherapy. Wang *et al.* postulated that activation of the Wnt pathway may protect salivary glands from radiation damage by several mechanisms, including inhibiting apoptosis. However, this same property has been postulated to contribute to the development of cancer, as well as leading to radioresistance of cancer stem cells (6). Wend *et al.* have demonstrated that the Wnt/β-catenin signaling pathway is involved in the development of rapidly growing, aggressive squamous cell carcinomas in the salivary glands of mice [9].

Similar to the Wnt pathway, the Notch pathway is involved in mediating developmental determination of cells. As demonstrated by Dang *et al.*, the Notch pathway is critical to the growth and differentiation of normal salivary gland cells (10). However, the Notch pathway has also been implicated in promoting inflammation, tumor angiogenesis, and tumorigenesis [10]. Bell *et al.* investigated the role of Notch signaling in adenoid cystic carcinoma and found elevated expression of both the Notch receptors and Notch ligand in AdCC relative to normal salivary gland tissue [8]. Ding *et al.* further demonstrated that Notch receptors were upregulated in a highly metastatic adenoid cystic carcinoma cell line compared to an adenoid cystic carcinoma cell line with low metastatic ability [7]. Delta-4 is an important ligand responsible for triggering the Notch signaling pathway.

In this study, the intensity of staining of various components of the Wnt and Notch signaling pathways was evaluated in both benign and malignant salivary tumors. Our observations correlate well with the findings from previous studies studies. FZD6, a component of the Wnt pathway, had significant expression in tumor tissue compared to normal salivary gland. Delta-4, a Notch receptor ligand, also had significant expression in tumor tissue compared to normal salivary gland. Both FZD-6 and Delta-4 had greater expression in malignant tumors and appear to play a larger role in tumorigenesis of aggressive subtypes of salivary gland tumors, particularly acinic cell carcinoma and adenoid cystic carcinoma respectively. Their expression was significantly elevated in aggressive type tumors as compared to slower-growing and lower grade tumor types.

β-catenin, a component of the Wnt pathway, was readily expressed in all tumors; however, it exhibits increased nuclear staining in more aggressive tumors compared to normal tissue and more benign tumor subtypes. Furuse *et al.* also demonstrated increased nuclear staining of β-catenin in epithelial-myoepithelial carcinomas (3). They hypothesized that the accumulation of cytosolic β-catenin accumulation

and subsequent nuclear translocalization results in constitutive Wnt/β-catenin signaling, and activation of transcription factors involved in cell cycle regulation. Nuclear translocation of β-catenin has been associated with aggressive behavior in salivary neoplasms, including adenoid cystic carcinoma [11].

FZD-6, β-catenin, and TGF-β proteins are involved in the Wnt signaling pathway, while Delta-4 is involved in Notch signaling; consistent with other studies, these pathways are thus implicated in growth patterns of aggressive and invasive salivary gland neoplasms. The Wnt and Notch signaling pathways have been extensively researched for their roles in the tumorigenesis of multiple neoplasms. Notch has been implicated as an important pathway in the development of T-cell acute lymphoblstic leukemia, chronic lymphocytic leukemia, lung, colorectal, ovarian, renal cell, and breast carcinomas. Similarly, the Wnt pathway is involved in the development of non-small cell lung cancer, ovarian cancer, osteosarcoma, and others. Identifying the role of the Wnt and Notch signaling pathways presents an opportunity to identify aggressive tumors earlier in their clinical course, which could lead to early treatment and improved outcomes. Additionally, understanding the role of Wnt and Notch signaling on the tumorigenesis of salivary tumors may lead to novel therapeutic targets and necessitates further research.

Conflict of Interest Statement

The authors of this study declare that they do not have any commercial or associative interest that represents a conflict of interest in connection with this work.

Acknowledgements

The authors would like to thank Susan MacDonald for collecting patient data. This study was made possible by a grant from RAM Capital.

References

1. Boukheris H, Curtis RE, Land CE and Dores GM. Incidence of carcinoma of the major salivary glands according to the WHO classification, 1992 to 2006: a population-based study in the United States. Cancer Epidemiol Biomarkers Prev. 2009, 18(11): 2899-06,

2. Guzzo M, Locati LD, Prott FJ, Gatta, G, McGurk M et al. Major and minor salivary gland tumors. Crit Rev Oncol Hematol. 2010, 74(2): 134-148,

3. Furuse C, Cury PR, Altemani A, dos Santos Pinto D, de Araujo MS et al. Beta-catenin and E-cadherin expression in salivary gland tumors. Int J Surg Pathol. 2006, 14(3): 212-217.

4. Zhao J, Li P, Feng H, Wang P, Zong Y et al. Cadherin-12 contributes to tumorigenicity in colorectal cancer by promoting migration, invasion, adhersion and angiogenesis. J Transl Med. 2013, 11: 288-299.

5. Gleber-Netto FO, Florencio TN, Desousa SF, Abreu MH, Mendonca EF et al. Angiogenesis and lymphangiogenesis in mucoepidermoid carcinoma of minor salivary glands. J Oral Pathol Med. 2012, 41(8): 603-609.

6. Wang JF, Liu C, Zhang Q, Huang GH. Research progress in the radioprotective effect of the canonical Wnt pathway. Cancer Biol Med. 2013, 10(2): 61-71.

7. Ding LC, She L, Zheng DL, Huang QL, Wang JF et al. Notch-4 contributes to the metastasis of salivary adenoid cystic carcinoma. Oncol Rep. 2010, 24(2): 363-368.

8. Bell D, Hanna EY, Miele L, Roberts D, Weber RS et al. Expression and significance of notch signaling pathway in salivary adenoid cystic carcinoma. Ann. Diag. Path. 2014, 18(1): 10-13.

9. Wend P, Fang L, Zhu Q, Schipper JH, Loddenkemper C et al . Wnt/β-catenin signalling induces MLL to create epigenetic changes in salivary gland tumours. EMBO J. 2013, 32(14): 1977-89.

10. Dang H, Lin AL, Zhang B, Zhang HM, Katz MS et al. Role for Notch signaling in salivary acinar cell growth and differentiation. Dev Dyn. 2009, 238(3): 724-731.

11. Daa T, Kaku N, Kashima K Nakayama I, Yokoyama S. Expression of beta-catenin, E-cadherin and cyclin D1 in adenoid cystic carcinoma of the salivary gland. J Exp Clin Cancer Res. 2005, 24(1): 83-87.